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A High-Titer Lentiviral Production System Mediates Efficient Transduction of Differentiated Cells Including Beating Cardiac Myocytes

Tsuyoshi Sakoda[1], Nori Kasahara[2], Yasuo Hamamori[1] and Larry Kedes[1]

[1]*Institute for Genetic Medicine and Department of Biochemistry & Molecular Biology, University of Southern California School of Medicine, Los Angeles, CA, USA;*
[2]*Institute for Genetic Medicine and Department of Pathology, University of Southern California School of Medicine, Los Angeles, CA, USA*

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T. SAKODA, N. KASAHARA, Y. HAMAMORI AND L. KEDES. A High-Titer Lentiviral Production System Mediates Efficient Transduction of Differentiated Cells Including Beating Cardiac Myocytes. *Journal of Molecular and Cellular Cardiology* (1999) 31, 2037–2047. Human immunodeficiency virus (HIV, lentivirus) type-1 based vectors have a number of attractive features for gene therapy, including the ability to transduce non-dividing cells and long term transgene expression. We used a three-plasmid expression system to generate pseudotyped lentivirus-based vectors by transient transfection of human embryonic kidney 293T cells in the presence of sodium butyrate, which is known to activate the long terminal repeat-directed expression of HIV. Using this system we successfully generated versatile high titer lentivirus at titers of up to 2×10^5 transducing units/ml (TU/ml), and improved transduction efficiency in various cell types from seven to over twenty fold. We demonstrate its applicability of these vectors for the efficient transduction of non-dividing cells, including post mitotic beating rat cardiac myocytes and well-differentiated rat L6 myofibers. While both lentivirus-based and murine retrovirus-based vectors effectively transduced dividing cardiac fibroblasts and L6 muscle myoblasts in culture, lentivirus-based vectors also efficiently transduced cardiac myocytes and yielded titers of $(6.3 \pm 1.2) \times 10^5$ TU/ml; however murine retrovirus-based vectors showed low transduction efficiency with titers reaching only $(8.9 \pm 2.1) \times 10^2$ TU/ml. Furthermore, even 12 days after induction of differentiation of L6 myofibers, lentivirus-mediated transduction of β-galactosidase (β-Gal) at approximately 30–40% of the maximum expression levels achieved in replicating myoblasts. In contrast, the expression of β-Gal following transduction of the myofibers by murine retrovirus-based vectors fell to less than 1% of an already reduced level of transduction in undifferentiated confluent myoblasts. These results demonstrate that lentivirus-based vectors can efficiently transduce both well-differentiated cardiac myocytes and differentiated myofibers. This appears to be an efficient method and provides a new tool for research and therapy for cardiovascular diseases.

KEY WORDS: Lentivirus; Sodium butyrate; Cardiac myocyte; L6 cell; Vesicular stomatitis virus; Retrovirus; Gene therapy.

Introduction

Gene therapy has the potential to reverse the genetic causes and modify the pathophysiology of many innate and acquired diseases (Mulligan *et al.*, 1993; Crystal *et al.*, 1995; Leiden *et al.*, 1995; Verma and Somia, 1997). Transduction of foreign DNA into cardiac myocytes or skeletal muscle myofibers is of potential value for therapeutic applications (Lafont *et al.*, 1996; Partridge and Davies, 1995) and also

Please address all correspondence to: L. Kedes, Institute for Genetic Medicine, University of Southern California School of Medicine, 2250 Alcazar Street, IGM240, Los Angeles, CA 90033, USA; e-mail: Kedes@hsc.usc.edu.

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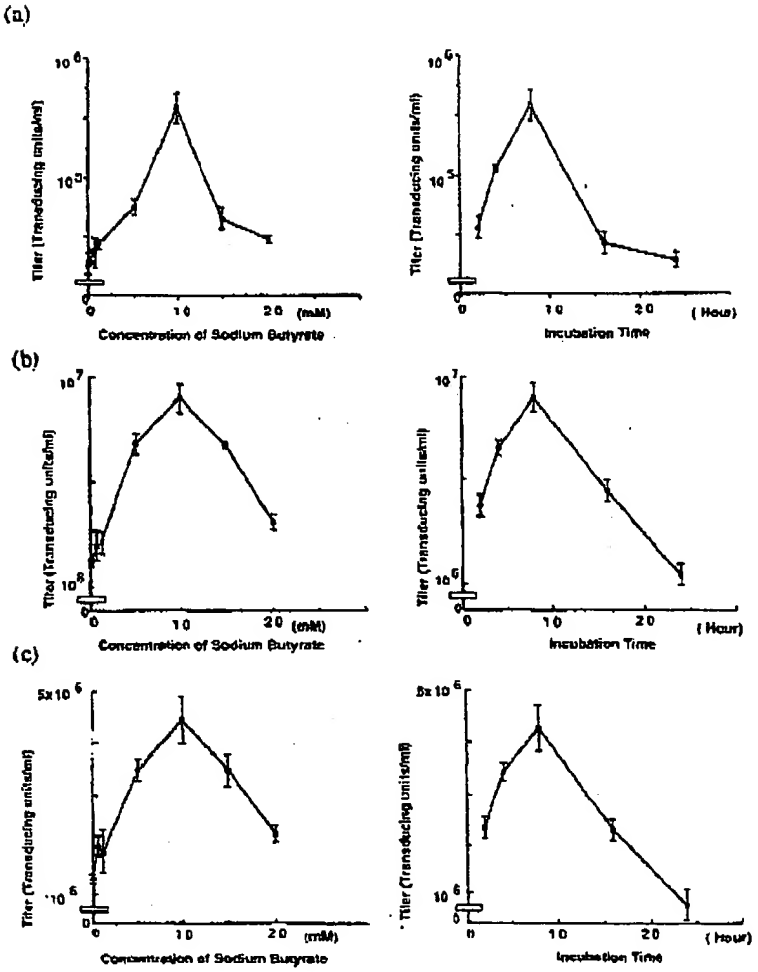

Figure 1 Effects of various concentrations and incubation times of sodium butyrate on titers (transducing units/ml, TU/ml) in various cells. (a) cardiac myocytes; (b) 293T cells; (c) L6 cells. Transduction is enhanced when 293T cells were exposed to sodium butyrate after transfection. Lentivirus-based vectors including GFP were generated by transient co-transfection of 293T cells. Sodium butyrate was administered at various concentrations and for various intervals. After replacing with fresh medium without sodium butyrate, conditioned medium was harvested, filtrated, and used for vector preparation. The values are means ± standard errors of three independent experiments.

offers an experimental approach to investigate the roles of individual genes in cardiovascular and muscle pathophysiology. Both efficient delivery and long-term expression of transduced genes is required before the full benefit of genetic manipulation strategies can be realized in the cardiovascular and muscular systems. However, current methods of gene delivery each suffer major limitations.

Non-viral methods of gene delivery remain inefficient and only attain transient expression of the transgene (Shi *et al.*, 1994). While adenovirus-based vectors allow highly efficient delivery of transgenes *in vitro* and *in vivo*, expression is transient because the transgene is not integrated in the host genome and immune responses against the transduced cells remain a concern (Schulick *et al.*, 1995). In contrast, standard retrovirus-based vectors, such as the Molony murine leukemia virus (Mo-MuLV), integrate the transgene into the genome of the target cells, which can sustain long term expression. However such retrovirus-based vectors can transduce only dividing cells (Lewis and Emerman, 1994), thus limiting their use in non-proliferating cells, especially differentiated cardiac myocytes and mature skeletal muscle myofibers. In addition, silencing of gene expression is a common

Table 1 Effect of optimum sodium butyrate exposure on lentiviral production

Cell Type	Sodium butyrate (−) Titer (transduction units/ml)	Sodium butyrate (+) Titer (transduction units/ml)	Fold change
Cardiac Myocytes	$2.5 (\pm 1.1) \times 10^4$	$6.3 (\pm 1.2) \times 10^5$	25.2
L6 Myoblasts	$0.38 (\pm 0.1) \times 10^6$	$4.25 (\pm 0.5) \times 10^6$	11.2
293T Cells	$1.3 (\pm 0.3) \times 10^6$	$9.0 (\pm 0.5) \times 10^6$	6.9

Cells were exposed to medium with (+) or without (−) 10 mM sodium butyrate for 8 hours and lentivirus-based vectors were harvested 16 hours after switching to medium without sodium butyrate.

occurrence. Recently, development of lentivirus-based vector systems based on a modified retroviral HIV genome has provided a delivery system that can both transduce non-dividing cells and integrate the transgene in the genome of target cells (Naldini *et al.*, 1996a). In addition, such lentivirus-based vector particles can be pseudotyped with the envelope of the vesicular stomatitis virus (VSV) (Burns *et al.*, 1993), thus enabling the vector to introduce genes into a broad range of tissues (Naldini *et al.*, 1996a,b; Miyake *et al.*, 1998). These features of lentivirus-based vectors, including their lack of cytotoxity, make them potentially useful for delivery of transgenes to cardiac myocytes and muscle myofibers. However there have been few reports regarding application of lentivirus-based vectors to cardiac myocytes and muscle myofibers (Rebolledo *et al.*, 1998; Kafri *et al.*, 1997; Mochizuki *et al.*, 1998) and these reports do not address the transducibility of such cells at various stages of differentiation compared with conventional retrovirus-based vectors. In this study we compare the degree of efficiency of lentivirus-based vectors with that of murine retrovirus-based vectors for transduction of cultured primary rat cardiac myocytes, cardiac fibroblasts, rat L6 proliferating myoblasts and differentiated multinucleated myofibers. Modification of the lentiviral particle production system, enabled us to increase viral particle titers more than 10-fold using sodium butyrate and to routinely generate titers of $>10^8$ TU/ml after one round of concentration. The derived lentivirus-based vectors carrying reporter genes (bacterial β-Gal (LacZ) or jellyfish green fluorescent protein (GFP)) efficiently transduced not only proliferating rat cardiac fibroblasts and L6 myoblasts, but also non-dividing cardiac myocytes and well-differentiated L6 myofibers. In contrast, murine retrovirus-based vectors transduced only dividing cardiac fibroblasts and L6 myoblasts, but did not transduce non-proliferating cardiac myocytes or differentiated myofibers. These findings suggest that lentivirus-bsed vectors should be useful for delivery of genes to the myocardium and skeletal muscle *in vivo*.

Materials and Methods

Cells and cell culture

Primary cultures of neonatal rat cardiac myocytes and cardiac fibroblasts were prepared as previously described (Susstman *et al.*, 1997; Ueyama *et al.*, 1997). The L6 rat skeletal muscle cell line was cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS). Differentiation was initiated by replacing the medium with DMEM with 2% horse serum (HS) (Yaffe, 1968). 293T human kidney cells as well as producer cells for the Mo-MuLV-derived BAG retrovirus which bears the β-Gal gene, were grown in DMEM supplemented with 10% FCS in a 37°C, 5% carbon dioxide humidified environment (Naldini *et al.*, 1996a,b; Price *et al.*, 1987).

Virus production and transduction

Lentivirus-based vectors encoding β-Gal or GFP were generated by transient co-transfection of 293T cells with a three-plasmid combination, as described previously, with slight modifications (Naldini *et al.*, 1996b). Briefly, a 100 mm dish of non-confluent 293T cells were transfected with 15 μg of pCMVΔR8.2, 15 μg of either pHR'-CMVLacZ or pHR'-CMVGFP and 15 μg of pMDG by the calcium phosphate DNA precipitation method (Chen and Okayama, 1987; Sakoda *et al.*, 1992). The plasmid vectors were kindly provided by Dr Luigi Naldini (Cell Genesys Inc., Foster City, CA). Sixteen hours after transfection, the media was adjusted to a final concentration of 10 mM sodium butyrate and the

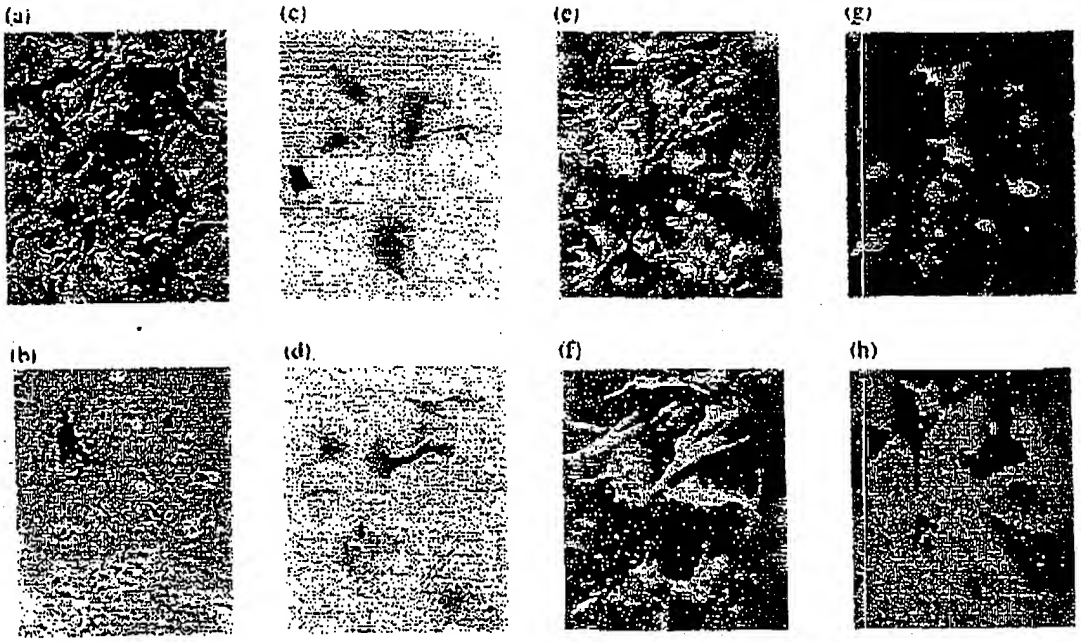

Figure 2

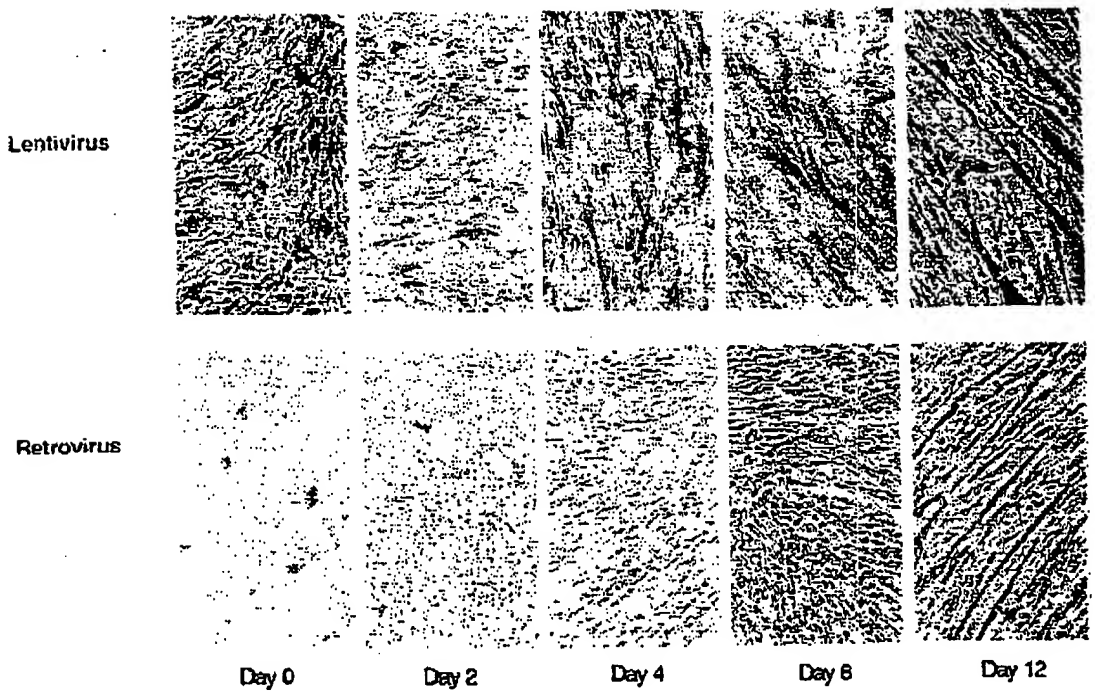

Figure 4

cells incubated for 8 hours unless noted otherwise in the text and figure legends. After the 8 hour incubation, cells were washed and incubated in fresh medium without sodium butyrate. Conditioned medium was harvested 16 hours later and filtrated through 0.45-μm filters. Concentrated viral stocks were prepared by one-round of ultracentrifugation of 3 ml of the conditioned medium at 50 000 g at 4°C for 90 minutes in an SW41 rotor (Beckman Instruments). The pellet was resuspended overnight at 4°C in 30 μl of Hanks balanced salt solution as described previously (Burns *et al.*, 1993). For transduction of cardiac myocytes, cardiac fibroblasts, L6 cells or 293T cells, cells were infected overnight with serial dilutions of virus stock in cultured medium supplemented with 8 μg of polybrene per ml. After medium replacement, the cells were incubated for 48 hours and expression of β-Gal or GFP was assessed. BAG retrovirus-based vectors carrying the β-Gal gene were prepared by harvesting supernatants from a stable producer cell line (Price *et al.*, 1987). Transductions were performed in the presence of 8 μg of polybrene per ml.

Analysis of transduced cells

Titers (TU/ml) were calculated by dividing the number of β-Gal or GFP expressing cells counted per dish by the dilution factor. Cells expressing β-Gal were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as described previously (Sanes *et al.*, 1986). Briefly, the cells were fixed with phosphate-buffered saline (PBS) containing 0.5% glutaraldehyde for 10 minutes at room temperature. After fixation, β-Gal expression was evaluated by histochemical staining with X-Gal in PBS containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6 3H_2O$, 1 mM $MgCl_2$ and 1 mg/ml X-Gal at 37°C for 16 hours. β-gal activity was measured using a β-galactosidase Enzyme Assay System (Promega) as directed by the manufacturer. In brief, transduced cells were washed with PBS and lysed in Reporter Lysis Buffer. 150 μl of these cell lysates were incubated with equal volume of Assay Buffer, which contains the substrate ONPG (o-nitrophenyl-β-D-galactopyranoside), for 30 minutes at 37°C. The absorbance of the samples at 420 nm was measured and β-Gal activities (μU/μg protein) were determined by a calibration curve. Protein concentrations were determined by the method of Bradford with bovine serum albumin used as a standard protein (Bradford *et al.*, 1976).

Rat cardiac myocytes were stained with anti-myosin monoclonal antibody MF20 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City) followed by Texas Red dye-conjugated goat anti-mouse immunoglobulin G (IgG). Briefly, isolated cardiac myocytes were fixed with 4% buffered formaldehyde for 5 minutes. The fixed cells were treated with 0.1% Triton X-100 for 2 minutes. After permiabilization in Triton X-100, cells were incubated for 1 hour with MF20, followed by incubation with Texas Red dye-conjugated goat anti-mouse IgG (1:333) for 1 hour at room temperature. Finally, the cardiac myocytes were washed several times in PBS and mounted on slides. Texas Red dye-conjugated anti-mouse IgG was purchased from Jackson Immuno Research Laboratories, Inc.

Figure 2 (*opposite*) β-Gal staining of cardiac myocytes and cardiac fibroblasts by lentivirus- or murine retrovirus-mediated β-Gal gene transduction. GFP fluorescence staining of cardiac myocytes by lentivirus-mediated GFP transduction and indirect immunofluorescence staining of cardiac myocytes using primary antisera against myosin (MF20). β-Gal, GFP fluorescence and indirect immunofluorescence staining were performed as described in Materials and Methods. (a) lentivirus-mediated β-Gal staining of cardiac myocytes; (b) murine retrovirus-mediated β-Gal staining of cardiac myocytes; (c) lentivirus-mediated β-Gal staining of cardiac fibroblasts; (d) murine retrovirus-mediated β-Gal staining of cardiac fibroblasts; (e) cardiac myocytes under bright field illumination—the same field as (f). (f) lentivirus-mediated GFP staining of cardiac myocytes; (g) lentivirus-mediated GFP staining of cardiac myocytes; (h) MF20 staining of cardiac myocytes—the same field as (g).

Figure 4 (*opposite*) Immunohistochemical staining for β-Gal of L6 cells by lentivirus- or murine retrovirus-mediated β-Gal gene transduction. L6 cells were grown in DMEM supplemented with 10% FCS until they reached confluency, at this point the medium was replaced by DMEM supplemented with 2% HS for induction of differentiation. Cells were transduced at indicate days after replacing with differentiation medium.

Results

Sodium butyrate increases production of lentivirus-based vectors

293T cells were transfected with pCMVΔR8.2, pMDG and pHR'-CMVGFP to produce lentivirus-based vectors encoding GFP. To examine the effects of sodium butyrate on virus production, cells were exposed to sodium butyrate at various concentrations and times starting 16 hours after transfection. At the end of this treatment, cells were replaced with fresh medium without sodium butyrate and the medium harvested 16 hours later.

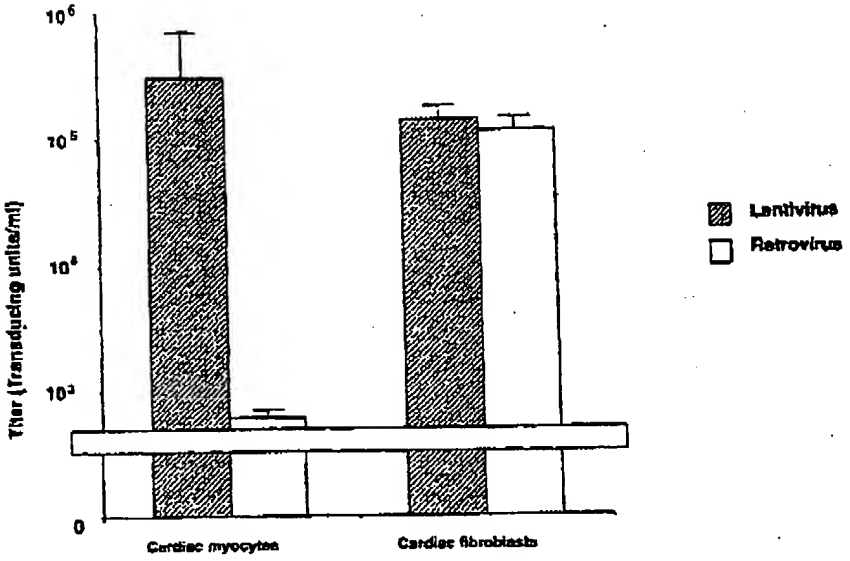

Figure 3 Comparative efficiency of lentivirus- and murine retrovirus-mediated β-Gal gene transduction in cardiac myocytes and cardiac fibroblasts. Titers were determined as described under Materials an Methods. The values are means ± standard errors of five independent experiments.

Figure 1 shows the effects of various concentrations and incubation times in sodium butyrate on the final viral vector titers. The viral titers were maximally enhanced when 293T cells were exposed to 10 mM sodium butyrate for 8 hours as determined by transduction of cardiac myocytes, 293T cells and L6 cells [Fig. 1(A–C)]. Sodium butyrate routinely increased titers in cardiac myocytes, 293T cells and L6 cells (Table 1). Thus, titers derived from 293T cells exposed to sodium butyrate were nearly one order of magnitude greater than those obtained without sodium butyrate. After only one-round of concentration by ultracentrifugation at 50 000 *g*, titers of $(2.2 \pm 1.0) \times 10^8$ TU/ml on 293T cells were routinely achieved.

Lentivirus-, but not murine retrovirus-based vectors mediate high efficiency gene transfer into well differentiated rat neonatal cardiac myocytes

Highly efficient transfer and stable expression of genes delivered to the myocardium or skeletal muscle remains an elusive goal. To determine whether lentivirus-based vectors can efficiently transduce both well differentiated cardiac myocytes and skeletal muscle myofibers, we carried out a series of gene delivery experiments with cells in culture and contrasted the transduction efficiencies of lentivirus- and murine retrovirus-based vectors. Primary neonatal rat cardiac myocytes and cardiac fibroblasts from the hearts of 2- to 3-day old rats were cultured for 3 days and then transduced with lentivirus-based or murine retrovirus-based vectors. The cells were stained with X-Gal or processed for immunofluorescence studies 3 days after the transduction. Figure 2(a) shows extensive β-Gal staining of cardiac myocytes by lentivirus-mediated LacZ transduction. While cardiac myocytes were successfully transduced by the lentivirus-based vectors, murine retrovirus-based vectors did not transduce the cardiac myocytes. The solitary β-Gal positive cell in Figure 2(b) is morphologically identified as a contaminating cardiac fibroblast. On the other hand, both lentivirus- and murine retrovirus-based vectors transduced cardiac fibroblasts [Figs 2(c) and (d) respectively]. Use of lentivirus-based vectors encoding GFP enabled us to assess transduction in living cells without fixation. Figure 2(e) and 2(f) show light and fluorescence confocal microscopy of such transduced cardiac myocytes. The GFP positive cells were beating cardiac myocytes. Finally, GFP positive cells clearly overlapped the Texas Red dye-positive myosin-expressing cells, indicating viral transduction of cardiac myocytes [Fig 2(g) and (h)]. The relative efficiencies of murine retrovirus- and lentivirus-based vectors on replicating cardiac fibroblasts and non-replicating cardiac myocytes are compared in Figure 3. In cardiac fibroblasts, both lentivirus- and murine retrovirus-

Table 2 Comparative efficiency of lentivirus- and retrovirus-mediated β-Gal transduction in L6 cells

Days after replaced to differentiation medium	Titer (transduction units/ml)	
	Lentivirus	Retrovirus
0	$1.45 (\pm 1.3) \times 10^6$ [100]	$8.95 (\pm 1.5) \times 10^5$ [100]
2	$8.53 (\pm 2.4) \times 10^5$ [58.8 ± 16.6]	$9.40 (\pm 2.5) \times 10^4$ [10.5 ± 2.79]
4	$6.63 (\pm 0.9) \times 10^5$ [45.7 ± 6.21]	$6.01 (\pm 1.9) \times 10^4$ [6.72 ± 2.12]
8	$5.96 (\pm 1.8) \times 10^5$ [41.1 ± 12.4]	$4.28 (\pm 2.3) \times 10^3$ [0.48 ± 0.26]
12	$5.25 (\pm 1.7) \times 10^5$ [36.2 ± 11.7]	$6.32 (\pm 2.1) \times 10^2$ [0.07 ± 0.02]

Cells were plated at low density and replaced by DMEM supplemented with 2% HS for induction of differentiation. Cells were transduced at indicated days after replacing with differentiation medium. Titers were determined as described under Materials and Methods. Results are also expressed relative to titer obtained in day 0. The values are means ± standard errors of three independent experiments.

based vectors effectively transduced and yielded similar titers of $(1.2 \pm 0.6) \times 10^5$ TU/ml and $(2.1 \pm 0.7) \times 10^5$ TU/ml, respectively. In cardiac myocytes, however only lentivirus-based vectors efficiently transduced with a titer of $(6.3 \pm 1.2) \times 10^5$ TU/ml. Furthermore, the GFP expression by lentivirus-based vectors persisted for at least 21 days with no apparent decrease (data not shown). The transduced cardiac myocytes showed no evidence of significant cytotoxicity by microscopic examination in line with previous reports (Naldini *et al.*, 1996*b*, Kafri *et al.*, 1997). In contrast, murine retrovirus-based vectors showed low transduction efficiency with titers reaching only $(8.9 \pm 2.1) \times 10^2$ TU/ml. These results demonstrate that efficient long-term transduction of cardiac myocytes *in vitro* can be achieved by lentivirus-based, but not murine rerovirus-based vectors.

Lentivirus-, but not murine retrovirus-based vectors mediate high efficiency gene transfer into differentiated L6 rat skeletal myofibers

Murine retrovirus-based vectors can efficiently transduce proliferating myoblasts but not differentiated myofibers. We tested the hypothesis that, unlike the retrovirus-based vectors, lentivirus-based vectors would retain the ability to transduce skeletal muscle cells following differentiation either as low density myoblasts or as fused multinucleated myofibers.

To determine transduction titers of lentivirus- or murine retrovirus-based vectors in L6 cells, the cells were plated at a low density (1×10^5 cells/6 cm dish) in DMEM supplemented with 10% FCS. The media was subsequently replaced by DMEM with 2% HS for induction of differentiation (Yafee, 1968). Viruses were transduced at 0, 2, 4, 8 or 12 days after switching to differentiation medium. The cells were stained with X-Gal 3 days after the transduction. Table 2 shows the comparative efficiency of lentivirus- and murine retrovirus-mediated β-Gal gene transduction of the L6 cells. Results are also expressed as absolute titers as well as relative to titers obtained at day 0. When the cells were plated at a low density and switched to differentiation medium, they become elongated but not fused, and remained as distinctive individual cells even at day 12. Lentivirus-based vectors expressing β-Gal maintained a relatively high transduction efficiency even at day 12 (36.2 ± 11.7%), whereas the efficiency of transduction by murine retrovirus counterparts fell to only 0.07 ± 0.02%.

We next compared the ability of lentivirus- and murine retrovirus-based vectors to transduce differentiated L6 myofibers. L6 cells were grown in DMEM supplemented with 10% FCS until they reached confluency. At this point, the medium was replaced with DMEM supplemented with 2% HS for induction of differentiation. Viruses were transduced at 0, 2, 4, 8 or 12 days after switching to differentiation medium. The cells were stained with X-Gal 3 days after transduction. As shown in Figure 4, lentivirus-based vectors can also efficiently transduce not only myoblasts (i.e. day 0) but also well differentiated myofibers (i.e. day 12). However, murine retrovirus-based vectors showed low transduction efficiency of myoblasts when confluency even at day 0. Moreover, in differentiated myofibers,

murine retrovirus-mediated β-Gal gene transduction was not observed. The comparative efficiency of lentivirus- and murine retrovirus-mediated β-Gal gene transduction for L6 cells is plotted in Figure 5.

Confluent L6 myoblasts transduced with lentivirus-based vectors encoding β-gal produced 188 ± 2.3 μU/μg protein at day 0 and maintained 30–40% of this level (69 ± 10.6 μU/μg protein) even when transduced at day 12. However, the expression of β-Gal following transduction by murine retrovirus-based vectors was only 37.5 ± 5.1 μU/μg protein at day 0 and fell to less than 1% (0.31 ± 0.67 μU/μg protein) at day 12 after differentiation. These results indicate that lentivirus-based vectors can efficiently transduce muscle cells at all stages of differentiation.

Discussion

We have developed an efficient system to enhance production of lentivirus-based vectors using sodium butyrate. Although much has been learned about the action of sodium butyrate upon cells, the molecular mechanisms of these effects remain unclear. Sodium butyrate produces a wide variety of effects on cells including the inhibition of histone deacetylase. Active transcription complexes or a modified chromatin structure, possibly via sodium butyrate-mediated acetylation of cellular histones has been suggested (Roman, 1982). There is considerable evidence for the enrichment of acetylated histones in transcriptionally active chromatin (Weisbrod, 1982). Accumulation of highly acetylated histones are required for efficient transcription (Grunstein, 1997).

With respect to the effect of sodium butyrate on virus production, sodium butyrate treatment has been reported to stimulate human cytomegalovirus (CMV) gene expression and viral replication in human endothelial cells (Radsak *et al.*, 1989). This enhancing effect by sodium butyrate on virus replication is not specific for human CMV. Similar results have been obtained using other human herpesviruses, Epstein-Barr virus (Saemundsen *et al.*, 1980) and herpes simplex virus (Ash, 1986). In addition, sodium butyrate has been reported to activate the long terminal repeat-directed expression of HIV (Bohan *et al.*, 1987). Therefore, sodium butyrate appears to be associated with a general induction of viruses. Whether these effects operate through some host cellular factors more efficiently transcribed when histones are highly acetylated or the acetylation of host transcription factors themselves remains unknown.

In this study, we analysed the effect of sodium butyrate on production of lentivirus-based vectors. Sodium butyrate treatment at an optimal concentration of 10 mM for an optimal duration of 8 hours increased titers by about 9-fold in 293T cells, 11-fold in L6 cells and 25-fold in cardiac myocytes compared to those obtained without sodium butyrate (Fig. 1 and Table 1). Thus, sodium butyrate clearly enhanced viral titers and increased the efficiency of transduction in all cell types tested. In these experiments, we harvested viruses from conditioned medium 16 hours after sodium butyrate withdrawal. Though the half-life of lentiviral particles under these conditions is only 4–6 hours, the effect of sodium butyrate on virus production was continued after its withdrawal. It has been reported that sodium butyrate-activated transcriptional state is continued after withdrawing sodium butyrate from the culture medium and can be imprinted in daughter cells for many generations (Tang and Taylor, 1992). Sodium butyrate induction of the Moloney murine sarcoma virus enhancer promoter-induced transcription was shown to be propagated from mother to daughter cells after withdrawal of sodium butyrate.

The present study also demonstrates that lentivirus-based vectors can efficiently transfer and express transgenes in beating cardiac myocytes and differentiated L6 myofibers. Skeletal muscle is an excellent target for somatic cell gene therapy. Transgene expression in skeletal muscle can be easily accessed and its large mass of muscle allows repeated administration of the recombinant vectors. Introduction of genetic material into muscle fibers has become of great interest not only for its potential therapeutic values for genetic diseases of muscle such as Duchenne muscular dystrophy or myotonic dystrophy, but also for the conversion of skeletal muscle to the production of systematically active gene products unrelated to muscle function (Hamamori *et al.*, 1995). *In vivo*, skeletal muscle contains a mixture of myogenic cell types, including muscle stem cells (satellite cells), proliferative myoblasts and differentiated myofibers, whose relative numbers vary depending on the state of muscle maturiy or processes such as repair or regeneration after injury. Therefore, gene transfer into muscle necessitates transduction at various stages of cell growth and differentiation with high efficiency and low toxicity. Cardiac myocytes on the other hand, are permanently withdrawn from the cell cycle soon after birth. Subsequently, myocardial growth is accomplished primarily by enlargement of

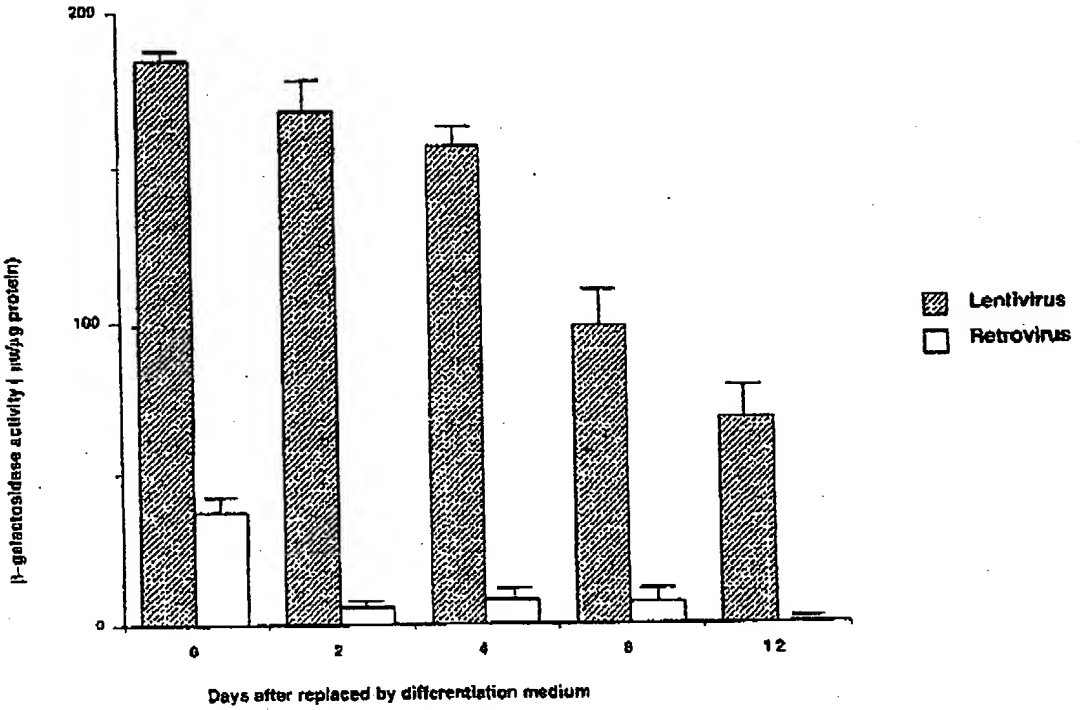

Figure 5 Comparative efficiency of lentivirus- and murine retrovirus-mediated β-Gal gene transduction in L6 cells. Cells were grown in DMEM supplemented with 10% FCS until they reached confluency, at this point the medium was replaced by DMEM supplemented with 2% HS for induction of differentiation. Viruses were transduced at the indicated days after induction of differentiation. As assessment of transduced cell counts was difficult with fused myofibers, cell lysates were prepared and β-Gal activity assayed 3 days after transduction. The results are expressed as β-Gal activity, *mU*/μg protein. The values are means ± standard errors of three independent experiments.

individual cardiac myocytes (i.e. hypertrophy), not by hyperplasia. Loss of cell in the myocardium following injury such as ischemia or cardiomyopathy, is not compensated by cell proliferation. Subsequently loss of myocardial mass can be a major cause of heart failure. For these reasons, for gene transfer to offer therapeutic potential to improve and maintain cardiac function, it cannot be accompanied by additional toxicity for the residual viable muscle into the heart, which cannot be well tolerated, but will rely on long-term expression with high efficiency.

Impressive gains have been made in delivery of genes to cardiac and skeletal myocytes using a variety of vectors. Although adenovirus-based vectors have been shown to be capable of transducing muscle cells *in vivo* and *in vitro* (Quantin *et al.*, 1990), gene expression was transient. In addition, standard E1, E3-deleted adenovirus-based vectors may have some direct cytopathic effects and induce immunological responses to transduced cells, because leaky expression of adenoviral genes can not be completely eliminated (Schulick *et al.*, 1995). Retrovirus-based vectors derived from Mo-MuLV are the most commonly used vectors in current clinical human gene therapy trials (Mulligan, 1993). Retroviruses integrate into chromosomal DNA thus providing potential for long-term expression of the transduced gene. However, it has been demonstrated that passage of target cells through mitosis is required for efficient transduction (Lewis and Emerman, 1994). This requirement for cell division greatly limits the use of murine retrovirus-based vectors for therapeutic gene transfer, especialy in non-replicating, differentiated cardiac and skeletal muscle myocytes. As an attractive alternative, lentivirus-based vectors pseudotyped with the VSV envelope have recently been reported to transduce a variety of cell types and this vector system also can transduce both dividing and non-dividing cells (Naldini *et al.*, 1996a,*b*). Furthermore, cytopathic or immunogenic effects of lentivirus-based vector itself have not been reported. In this study, we examined the transduction efficiency of

lentivirus-based vectors dividing and non-dividing cells and compared with murine retrovirus-based vectors. Our results show that unlike retrovirus-based vectors, lentivirus-based vectors can transduce genes efficiently into non-dividing cardiac myocytes and L6 myofibers at various stages of differentiation. As lentivirus-based vectors also integrate in the genome of the target cells (Naldini *et al.*, 1996a,*b*; Miyake *et al.*, 1998), repeated transduction is unnecessary. In consequence, humoral responses to repeat injections of viral antigens can be avoided (Knowles *et al.*, 1995), in contrast to adenovirus-based vectors. Moreover, the lentivirus-based vectors studied in this paper are completely replication-defective (Naldini *et al.*, 1996*a*), consequently, the transduced cells will not express viral proteins that might trigger cellular immune responses. The *in vitro* results from the present study provide important information for *in vivo* application of lentivirus-based vectors to cardiac and skeletal muscle. The availability of integrating vectors that can deliver genes to heart and muscle without evoking immune responses sets the stage for further studies of cardiovascular and neuromuscular gene therapy.

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Results

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